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Guide to Making Useful Measurements of Monoclonal Antibodies (mAbs) with Dynamic Light Scattering

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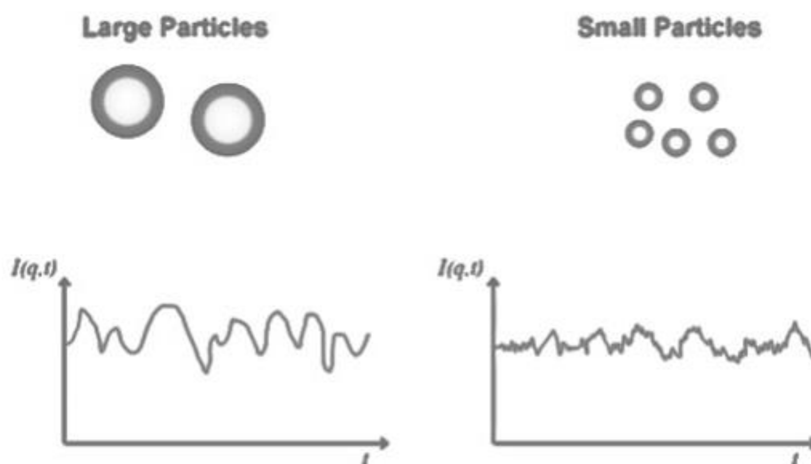
Dynamic light scattering (DLS) is a powerful technique that is amenable to measurement of biological samples, particularly proteins and biopolymers. One of the fastest growing classes of pharmaceutically active biologics are antibodies. These highly versatile proteins are used as the major functional element in immunoassays and other rapid diagnostics, in vaccine production, and as the primary component of a wide array of injectable protein drugs. In all these applications it is essential that antibodies remain intact, and monomeric. Failing to adhere to these strict and exacting standards compromises the processability, activity, and shelf stability of antibody-based products. When DLS is used to its full potential, aggregation of proteins can be detected and the size of monomeric protein can be determined to a very high degree of certainty. In order to demonstrate this principle, we compare aggregation studies conducted on pharmaceutical-grade monoclonal antibody (mAb), with results obtained for a common protein, serum albumin (BSA).

Introduction:

DLS relies on the principle that freely diffusing material, moving randomly due to Brownian motion, will produce rapid fluctuations in scattered laser light. The timescale of these intensity fluctuations is on the order of tens of nanoseconds to hundreds of milliseconds. These fluctuations are directly related to the motion of particles. The signal that arises from the scattered intensity from the laser light is acquired and transformed into an autocorrelation function. *This is the basis for measuring a particle size distribution.*



Brookhaven Instruments
750 Blue Point Road
Holtsville, NY 11742
Phone: +1 (631) 758-3200
Fax: +1 (631) 758-3255
E-mail: info@brookhaveninstruments.com
Web: brookhaveninstruments.com

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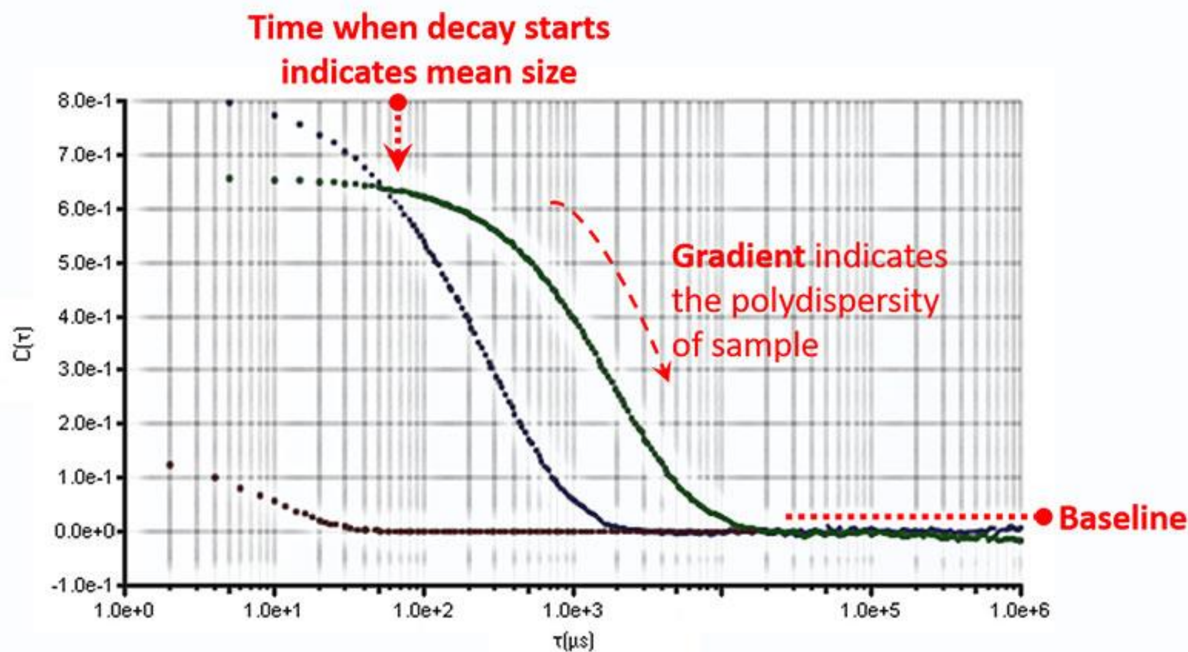

Many common globular proteins are very small in hydrodynamic size. Protein monomers are typically < 10 nm, with many as small as 1 nm or less. A high-speed correlator is necessary to measure the size of such rapidly diffusing proteins. Protein aggregates can easily reach large sizes on the order of several hundred nanometers or greater. A typical sample comprising partially aggregated protein might span 1-2 orders of magnitude in hydrodynamic size. The challenge in this case is choosing the appropriate correlator layout to fully resolve these signals. As will be shown below, the *configurability* of the correlator is key to measuring otherwise difficult heterogeneous biological samples.

It is important to note that the diameter obtained from DLS, often referred to as the hydrodynamic diameter ($\mathbf{d_h}$), is inversely proportional to the diffusion coefficient ($\mathbf{D_t}$). The relationship between $\mathbf{D_t}$, measured in DLS, and hydrodynamic size, $\mathbf{d_h}$, is inverse, and is given by the Stokes-Einstein Equation:

$$\mathbf{D_t} = k_B T / 3\pi\eta \mathbf{d_h}$$

Where the Boltzmann constant (k_B), temperature (T), and bulk viscosity (η) are all known values. This expression can be simplified to a proportionality:

$$D_t \propto 1/d_h$$

The convention in light scattering is to refer to small scattering angles as forward-scatter, and large-scattering angles as backscatter. Backscatter, typically referring to angles much larger than 90°, is very commonly used to measure the sizes of small, monomer-sized proteins. Forward-scatter, on the other hand, is overly sensitive to larger species, and can be used to detect the presence of even a small amount of aggregated protein. For a known scattering angle, θ, and refractive index, n, the scattering vector **q** is calculated from the following expression:

$$\mathbf{q} = 4\pi n/\lambda_o \sin(\boldsymbol{\theta}/2)$$

Brookhaven Instruments
750 Blue Point Road
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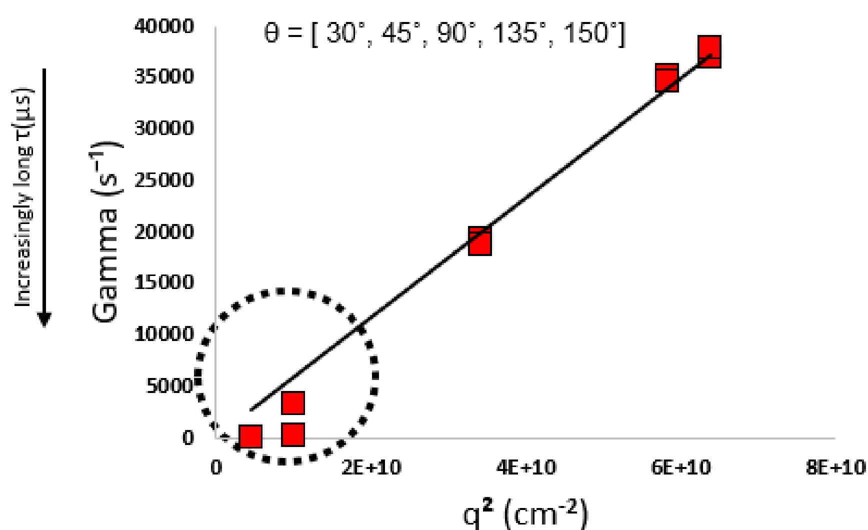
Fax: +1 (631) 758-3255
E-mail: info@brookhaveninstruments.com
Web: brookhaveninstruments.com

A given correlation function C(τ) is deconvoluted into either a single-exponential, stretched-exponential, or sum of exponentials. The different components of this function can be separated into a characteristic decay rate, Γ, and typically also a polydispersity index. This Γ is related to the translational diffusion coefficient (D_t) as follows:

$$\Gamma = D_t q^2$$

Results & Discussion:

For a typical medium-sized globular protein, decay rates (Γ) as low as several hundred s^{-1} and as high as 50000 s^{-1} may be observed, depending on scattering angle. A configurable correlator layout enhances the ability to measure such a broad range of decay rates. As shown below for BSA, Γ depends linearly on q^2 for a particle of a given size. In most common applications, DLS at a single angle can be used to solve for D_t, and thus to obtain the diameter of the protein in question. Common DLS configurations, particularly benchtop instruments, tend to have backscatter, right-angle, and forward-scatter (θ = 173°, 90°, and 15°).



$$\Gamma = D_t q^2$$

D_t = 5.7x10^{-7} cm^2/sec
d_h^{eff} = 7.5 nm

DLS results for serum albumin monomer as a function of scattering angle, θ. Effective d_h = 7.5 nm. For this Γ vs q^2 dependence to be useful, a minimum of 4-5 scattering angles is required.

Backscatter can be used to measure the size of protein monomers. Generally, backscatter angles are useful for measuring samples in which there is a mixture of free and aggregated protein. Backscatter angles minimize the intrinsic intensity bias towards larger particles, which would otherwise obscure scattering from monomeric protein.

Forward-scatter is highly sensitive to aggregation. In contrast to backscatter angles, very low angles are highly sensitive to large particles especially those much greater than 1000 nm. While forward-scatter is highly sensitive to presence of

Brookhaven Instruments
750 Blue Point Road
Holtsville, NY 11742
Phone: +1 (631) 758-3200
Fax: +1 (631) 758-3255
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Web: brookhaveninstruments.com

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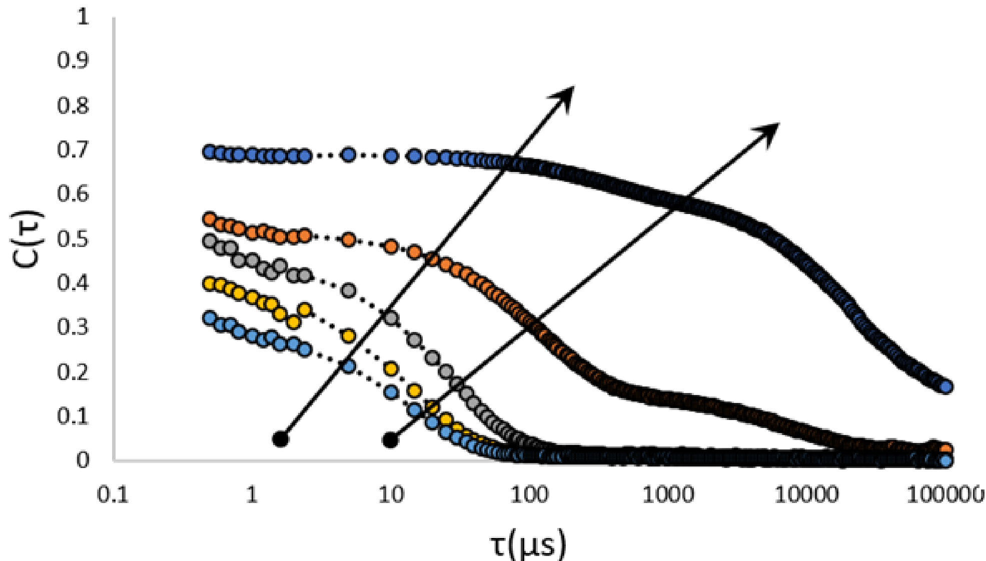
aggregates, backscatter is more useful for accurately determining the size of any small particles that may coexist with aggregated material.

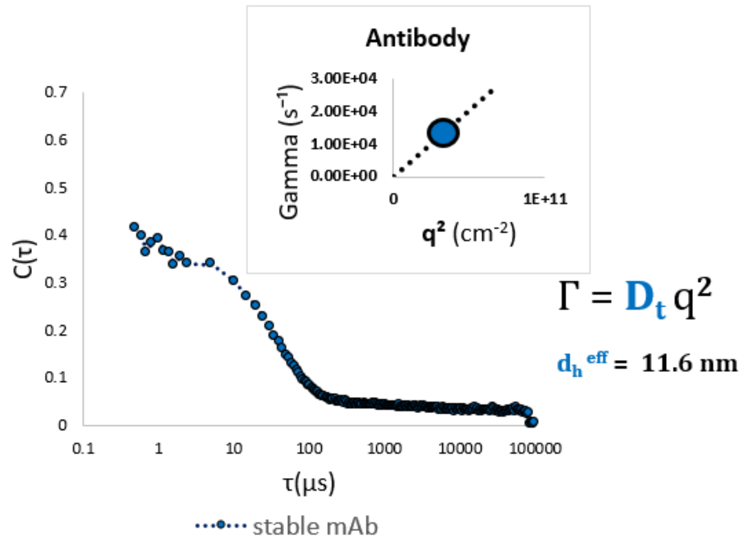


Correlation functions obtained at five separate scattering angles, θ = (30,150°). The existence of a second decay becomes obvious at the two lowest angles.

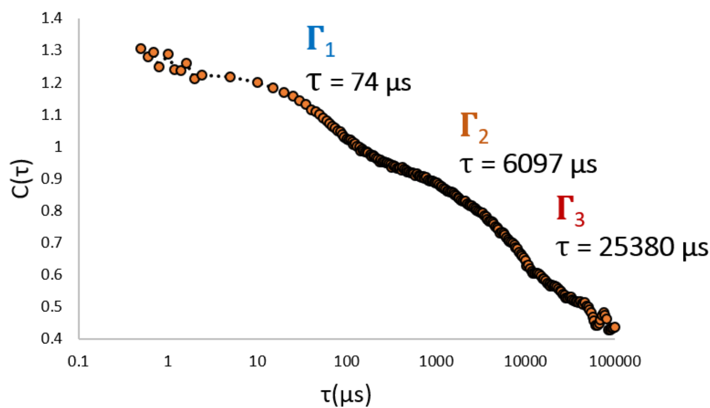
Serum albumin appears monomeric when measured in backscatter. If one were to make a DLS measurement only at backscatter angles, the scattering of BSA could easily be mistaken for that of pure monomer. The existence of a second decay only becomes obvious at the lowest angles (30 ° and 45°). Recall that a shift towards slower diffusion, or smaller Γ, indicates that larger particles are present. This suggests that large, slow moving particles, possibly aggregated protein, must coexist with monomeric protein.

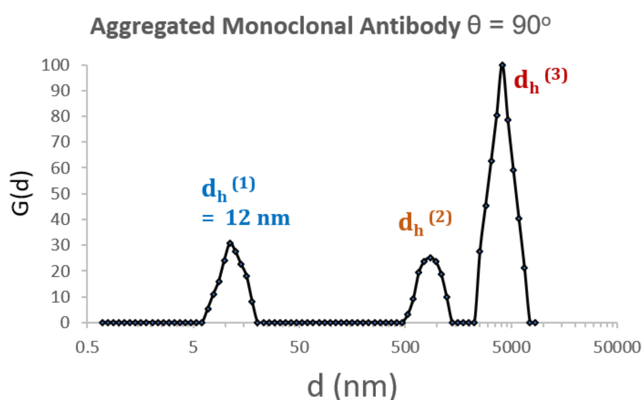
BSA is a conventional serum protein and is often used as a stand-in for more pharmaceutically relevant proteins such as antibodies. This compact, globular protein is known to exist in monomeric, dimeric, and occasionally trimeric forms. At physiological pH, a very small percentage of aggregated BSA might be expected. In contrast, antibodies are large, flexible proteins, with compact subunits, held together by numerous, fragile, internal disulfide bonds. Chemically degraded antibodies can either be expected to aggregate, or to produce low molecular weight antibody fragments.

Monomeric mAb can be measured easily using a single angle. As shown below, stable monomeric monoclonal antibody can be measured effectively at a single scattering angle, θ = 90°. This measurement yields an effective diameter of around 12 nm, and a low, near zero, polydispersity. The correlation function is best described by a single exponential decay, suggesting that the sample is easily characterized and homogeneous, with no aggregate detected.



Dilute monoclonal antibody measured at a ninety-degree scattering angle shows a single effective diameter of around 11.6 nm.





$\Gamma_1 = 1.35\text{x}10^4\ s^{-1}$

$\Gamma_2 = 1.64\text{x}10^2\ s^{-1}$

$\Gamma_3 = 3.94\text{x}10^1\ s^{-1}$

DLS of chemically stressed monoclonal antibody produces a correlation function with 2-3 major components, where the dominant component is aggregated protein.

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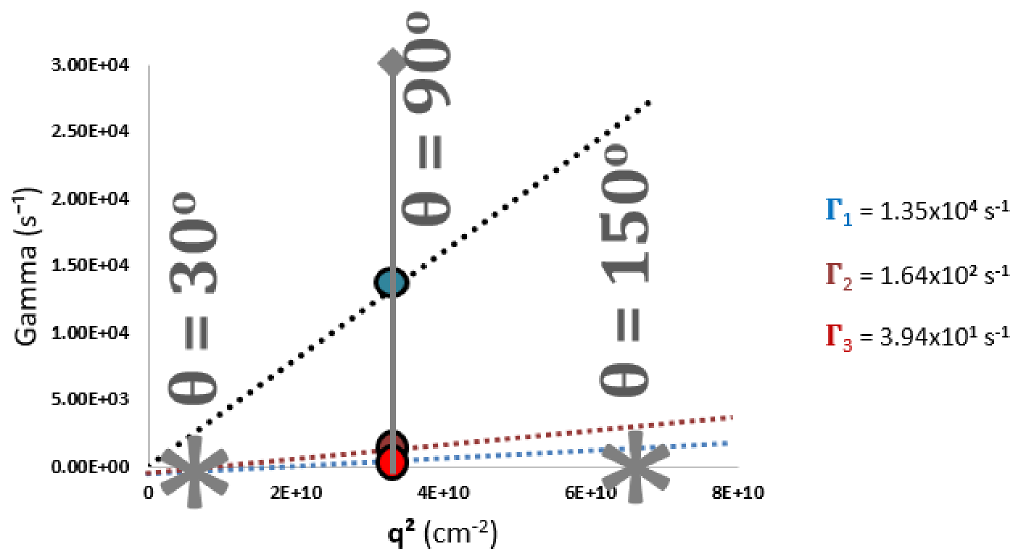
Web: brookhaveninstruments.com

It is clear that this sample (above) contains multiple, discrete and well-defined populations, as would be expected for a sample containing a mixture of aggregated and free protein. In contrast, chemically stressed antibody cannot be described by a model containing a single decay constant, and it cannot be meaningfully described as a continuous distribution of particle sizes. Since these Γ's span multiple orders of magnitude in s^{-1}, we can resolve them only by carefully selecting a correlator layout that covers a wide range of delay times (high, medium and low speed channels).

Free monomeric antibody peaks are fully resolvable from aggregate peaks when using a multimodal particle size distribution, provided that an appropriate scattering angle ($\theta \geq 90^o$) is used. It is apparent that our choice of a large scattering angle (below) improves our ability to resolve multiple rapid decays.



Resolvability of multimodal distribution as a function of scattering angle, θ, and scattering vector, q^2.

Careful choice of both correlator layout, including range of delay times (τ), and scattering angle allow for monomeric mAb to be measured with a high degree of precision, even in the presence of heavily aggregated protein. Note the difference in resolvability of Γ_1, Γ_2, Γ_3, at 15° vs. 155 °.

RECAP:

- Selecting an optimal scattering angle is key to accurately measuring challenging biological samples.
- Continuous multi-angle measurements can be informative, especially when starting with unknown mixtures of aggregated and monomeric protein. However, this is often unnecessary for stable, homogenous protein samples.
- Recall that forward-scatter refers to very small angles, whereas backscatter refers to high angles. Benchtop instruments commonly have three fixed scattering angles, forward, right-angle, and backscatter.

- **Key points:**
  - ✓ Backscatter is useful for high-precision measurement of small particles or mixtures.
  - ✓ Low angles are very sensitive to larger particles; this is why forward-scatter is useful for detecting even small numbers of protein aggregates.
  - ✓ Forward-scatter angles, while useful in detecting aggregation, are less helpful when it comes to quantifying the sizes of multiple coexisting species.

Conclusions:

Protein aggregates can easily reach large sizes, on the order of several hundred nm or larger, which complicate this already challenging measurement. This principle is demonstrated with a pharmaceutically relevant protein, monoclonal antibody (mAb), studied in both its monomeric and aggregated states. Despite its heterogeneous nature, careful selection of both correlator layout and scattering angle allows us to fully resolve the signal from monomeric mAb, even in the presence of chemically induced aggregates.

Applications: *DLS, Proteins, Monoclonal Antibodies, Biopharma, Biotech, Particle Sizing*
Instruments: *BI200SM, NanoBrook*
URL: brookhaveninstruments.com/guide-to-making-useful-measurements-of-monoclonal-antibodies-mabs-with-dynamic-light-scattering/